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MDM2 Gene Amplification and Expression in Non-Small-Cell Lung Cancer Patients*

Amplifikacja i ekspresja genu *MDM2* u chorych na niedrobnokomórkowego raka płuca

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Abstract

Background. The authors recently demonstrated the adverse prognostic impact of *MDM2* gene amplification, but not mdm2 protein expression, in non-small-cell lung cancer (NSCLC) patients.
Objectives. To understand this discrepancy, the authors investigated the correlation between *MDM2* gene amplification and the expression of its protein product in 83 NSCLC patients who underwent curative pulmonary resection.
Material and Methods. *MDM2* gene amplification was assessed by real-time PCR on a LightCycler (Roche) using the hybridization probe format. mdm2 protein expression was assessed immunohistochemically with the use of monoclonal antibody (IF2, Oncogene Science) and the APAAP technique. Any nuclear expression of mdm2 protein was considered positive.
Results. *MDM2* gene amplification was found in 15 of the 83 NSCLC patients (18%), mdm2 protein expression in 35 patients (42%), and both alterations in 7 (8%). There was no correlation between *MDM2* gene amplification and the expression of its protein product ($p = 0.70$) nor between *MDM2* gene amplification/expression (considered separately or jointly) and patient characteristics.
Conclusions. These results suggest that mdm2 protein accumulation in NSCLC cells does not necessarily result from *MDM2* gene amplification, but might also be related to other mechanisms, such as increased transcription of MDM2mRNA or enhanced mdm2 protein translation (**Adv Clin Exp Med 2006, 15, 4, 589–593**).

Key words: *MDM2* gene, mdm2 protein, lung cancer.

Streszczenie

Wprowadzenie. W poprzednim badaniu zespołu autorów wykazano niekorzystne znaczenie rokownicze amplifikacji genu *MDM2* (w odróżnieniu od ekspresji białka mdm2) u chorych na niedrobnokomórkowego raka płuca (n.d.k.r.p.).
Cel pracy. Aby wyjaśnić przyczynę powyższej rozbieżności, oceniono zależność między amplifikacją genu *MDM2* a ekspresją jego białkowego produktu w grupie 83 chorych operowanych z powodu n.d.k.r.p.
Materiał i metody. Materiał do oceny amplifikacji genu *MDM2* stanowiło DNA wyizolowane z tkanek niedrobnokomórkowego raka płuca. Analizę przeprowadzono z użyciem techniki łańcuchowej reakcji polimerazy (PCR) z zastosowaniem cyklera świetlnego. Materiał do oceny występowania białka mdm2 stanowiły fragmenty guzów n.d.k.r.p., utrwalone w formalinie i przechowywane w postaci bloków parafinowych. Obecność białka mdm2 ba-

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dano immunohistochemicznie, z użyciem przeciwciała monoklonalnego IF2 (Oncogene Science) oraz metody APAAP. Za dodatnie uznano guzy, które wykazywały jądrową ekspresję białka mdm2.
Wyniki. Amplifikację genu *MDM2* stwierdzono u 15 spośród 83 chorych (18%), a obecność białka mdm2 – u 35 chorych (42%). W 7 przypadkach (8%) stwierdzono jednoczesne występowanie amplifikacji genu *MDM2* oraz białka mdm2. Nie stwierdzono zależności między występowaniem amplifikacji genu *MDM2* i nagromadzeniem się jego białkowego produktu (p = 0,70). Nie stwierdzono również zależności między amplifikacją/ekspresją genu *MDM2* (analizowanymi pojedynczo lub łącznie) a cechami klinicznymi chorych.
Wnioski. Wydaje się, że w komórkach n.d.k.r.p. obecność białka mdm2 nie wynika wyłącznie z amplifikacji genu *MDM2* i może być rezultatem bardziej złożonych mechanizmów (**Adv Clin Exp Med 2006, 15, 4, 589–593**).

Słowa kluczowe: gen *MDM2*, białko mdm2, rak płuca.

Lung cancer is the most common human malignancy worldwide and its incidence is increasing [1]. Non-small-cell lung cancer (NSCLC) amounts to more than 80% of all lung cancers. Although considerable therapeutic progress has been made, the prognosis of NSCLC patients is still unsatisfactory [2]. Surgical resection remains the therapy of choice in the early stages of disease; however, only 15–25% of all NSCLC patients are candidates for surgery. Moreover, even in this selected population about half of the patients will relapse after complete resection [2]. It is believed that a better understanding of lung cancer biology may result in more efficient cancer management.

MDM2 protooncogene (12q13-14) encodes the 90-kDa mdm2 oncoprotein, which is physically associated with p53 protein [3]. mdm2 and p53 form an autoregulatory feedback-loop in which p53 positively regulates the mdm2 levels and mdm2 inhibits p53 expression and activity [4]. Recently it has been suggested that *MDM2* also has a p53-independent activity in cancer development [5, 6], but the molecular mechanisms of this process remain unknown [6]. The *MDM2* gene has been shown to be abnormally up-regulated in human tumors and tumor cell lines by gene amplification, increased transcript levels, and enhanced translation [7]. The frequency of *MDM2* amplification differs across particular malignancies. This alteration has been observed, for example, in 28% of soft tissue sarcomas, 33% of uterine sarcomas, 42% of liposarcomas, 18% of high-grade osteosarcomas, and 42% of gastric carcinomas [7]. There are only a few studies addressing *MDM2* alterations in NSCLC. In particular series, *MDM2* amplification occurred in 0 to 21% of cases [8–11], *MDM2* mRNA expression in 43% [12], and mdm2 protein overexpression in 6 to 78% [9, 11–16].

The prognostic value of *MDM2* gene alternations in NSCLC is controversial. In previous study, *MDM2* amplification correlated with shortened disease-free and overall survival in NSCLC patients [8]. In contrast to these findings, in another group of NSCLC patients the authors did not observe any prognostic impact of mdm2 protein expression [14]. To understand this discrepancy, in this study they decided to assess simultaneously *MDM2* gene amplification and protein expression in the same group of NSCLC patients.

Material and Methods

The study group consisted of 83 NSCLC patients who underwent curative pulmonary resection at the Department of Thoracic Surgery, Medical University of Gdańsk, Poland, between 1996 and 1999 (Table 1). None of the patients had undergone preoperative treatment for NSCLC. The database included age, sex, tumor histology and grade, pTNM designation, stage of disease, date and extent of surgery, adjuvant treatment, time and site of recurrence, survival status, smoking habit, and *MDM2* gene amplification and expression. Tissue specimens were taken intraoperatively and divided into two parts, one of which was

Table 1. Patient characteristics, n = 83

Tabela 1. Cechy kliniczne badanej grupy chorych, n = 83

Variable (Zmienna)	Number (Liczba)
Age (Wiek)	
≤ 60 years	39
> 60 years	44
Sex (Płeć)	
female	21
male	62
Stage (Stadium)	
I	31
II	10
IIIA	37
IIIB + IV	5
Histology (Histologia)	
squamous cell carcinoma	48
adenocarcinoma	20
large cell	8
mixed type	7
Tumor differentiation (Zróżnicowanie guza)	
G1	10
G2	50
G3	23

immediately frozen in liquid nitrogen and stored at –80°C, and the other fixed in formalin and embedded in paraffin. Hematoxylin-eosin-stained tissue sections were classified and differentiated according to the revised classification of the WHO [17]. Determination of the stage of disease (pTNM) was based on the current UICC criteria [18] after pathological examination of primary tumor and regional lymph nodes.

MDM2 gene amplification was assessed as previously described [8] by real-time PCR on a LightCycler (Roche) using the hybridization probe format. The calculated ratio was the *MDM2* value normalized to the amplification of the house-keeping gene *PAH*. Because positive control had a 20-fold *MDM2* gene amplification (ratio 1.5), linking this result to negative control (ratio 0.1) the authors calculated that a cut-off value of > 0.3 corresponds to more than four copies of *MDM2* gene [19]. Twenty percent of the distance between the mean ratio of the negative and positive control was defined as the cut-off value (ratio of 0.3), i.e. ratios ≤ 0.3 were considered negative and ratios > 0.3 positive. mdm2 protein expression was assessed as previously described [8] immunohistochemically with the use of monoclonal antibody (IF2, Oncogene Science) and the APAAP (alkaline phosphatase-antialkaline phosphatase labeling) technique. Any nuclear expression of mdm2 protein was considered positive.

Statistical analysis was done using STATISTICA 6.0 program. The chi-square test was used to assess the relation between *MDM2* amplification/expression and clinical characteristics, i.e. patient age, sex, stage of disease, and tumor type and differentiation.

Results

MDM2 amplification was found in 15 of the 83 NSCLC patients (18%). The mean ratio of *MDM2*-positive cases was 0.8 (range: 0.33–4.7). The mean ratio of *MDM2*-negative cases was 0.18 (range: 0.08–0.3). In all 83 patients, apart from *MDM2* amplification, the authors also assessed the expression of mdm2 protein immunohistochemically. mdm2 protein expression was found in 35 patients (42%) and both gene and protein alterations in 7 (8%). No correlation was found between *MDM2* amplification and expression by chi-square analysis ($p = 0.70$). There was also no correlation between *MDM2* gene amplification or mdm2 protein expression and patient age ($p = 0.93$ and $p = 0.43$, respectively), gender ($p = 0.49$ and $p = 0.85$), stage of disease ($p = 0.96$ and $p = 0.17$), and tumor type ($p = 0.53$ and $p = 0.11$) and differentiation ($p = 0.64$ and $p = 0.15$). Simultaneous analysis of *MDM2* gene amplification and expression did not show any correlation between the occurrence of both alterations and patient age ($p = 0.11$), sex ($p = 0.49$), stage of disease ($p = 0.68$), tumor type ($p = 0.91$) and differentiation ($p = 0.45$). Smoking was excluded from this analysis because 82 of the patients (99%) were current or previous smokers.

Discussion

In operable NSCLC, the only consistent prognostic factor is tumor stage. The prognostic value of novel tumor markers, such as DNA ploidy, proliferation markers, inactivation of tumor suppressor genes, and up-regulation of protooncogenes in

Table 2. *MDM2* gene amplification and expression in NSCLC patients

Tabela 2. Amplifikacja genu *MDM2* oraz ekspresja białka mdm2 u chorych na n.d.k.r.p.

	MDM2 amplification (Amplifikacja *MDM2*)		*MDM2* expression (Ekspresja *MDM2*)		*MDM2* amplification and expression (Amplifikacja i ekspresja *MDM2*)	
	number of included patients (liczba włączonych pacjentów)	number of positive patients (liczba pacjentów zweryfikowanych pozytywnie)	number of included patients (liczba włączonych pacjentów)	number of positive patients (liczba pacjentów zweryfikowanych	number of included patients (liczba włączonych pacjentów)	number of positive patients (liczba pacjentów zweryfikowanych pozytywnie)
Marchetti et al. [11]	53	3 (6%)	53	3 (6%)	53	3 (6%)
Higashiyama et al. [10]	30	2 (7%)	201	48 (24%)	30	2 (7%)
Gorgoulis at al. [9]	41	0 (0%)	41	26 (63%)	41	0 (0%)
Dworakowska et al. [8]	116	24 (21%)	36	12 (33%)	36	2 (6%)
Present study (Obecna praca)	83	15 (18%)	83	35 (42%)	83	7 (8%)

cancer cells, is still a matter of controversy [20]. The most common molecular alteration in lung cancer is mutation of the tumor suppressor gene *P53,* closely related to *MDM2*. The prognostic value of *P53* mutation and protein expression is questionable [21]. In a recent study the authors demonstrated a negative prognostic impact of *MDM2* gene amplification [8] (but not mdm2 protein expression) [14]. However, due to tissue limitations, both analyses were performed in separate groups of NSCLC patients (the overlap included 36 patients) [8].

There are only four studies addressing the joint analysis of *MDM2* gene amplification and protein expression in NSCLC [8–11] (Table 2). In previous study the frequency of *MDM2* gene amplification was 21% [8], which was higher than in other NSCLC series [9–11]. In that study, *MDM2* gene amplification and protein overexpression was assessed only in a group of 36 patients and they were found to be altered in 2 (6%) and 12 patients (17%), respectively [8]. In the present analysis the study group was increased to 83 NSCLC cases; however, no correlation between *MDM2* gene amplification and protein expression was found (both alterations occurred in 8% of patients). Marchetti et al. [11] found joint occurrence of *MDM2* amplification and protein expression in three of 53 NSCLC cases (6%) and Higashiyama et al. [10] in two of 30 cases (7%); however, mdm2 protein expression without *MDM2* gene amplification was observed in 24% of the analyzed cases. Gorgoulis et al. [9] did not find *MDM2* amplification in 41 NSCLCs despite the fact that mdm2 protein expression was present in 63% of cases. Similarly, in other types of cancers, for instance in breast cancer [22] and adult soft tissue sarcomas [23], mdm2 overexpression also did not always correlate with *MDM2* gene amplification.

MDM2 may be up-regulated by mechanisms other than *MDM2* gene amplification, including enhanced translation and gene translocation, though it is not clear whether these events occur in human tumors [7]. Indeed, in most of the studies addressing *MDM2* amplification and protein expression, the latter occurred more frequently [8–10]. *MDM2* transcript levels have been shown to be relatively high in several tumors, for example, leukemias and lymphomas, with no gene amplification. If mdm2 protein is overexpressed through another abnormal mechanism, it would suggest that gene amplification analysis leads to an artificially low frequency of *MDM2* involvement in human tumors. A simple model is that an *MDM2* promoter-specific transcription factor can be up-regulated. Such a factor would lead to direct inactivation of p53 [24]. The *MDM2* promoter is a direct target of p53. It is possible that some tumor cells exhibiting high levels of *MDM2* transcript may actually have functional p53. Some immunohistochemical studies showed a relative coincidence of high mdm2 and p53 levels. Therefore it is difficult to rule out the possibility that mdm2 protein overexpression results from normal p53 signaling in these tumors. [7]. However, in previous study the authors did not find any correlation between mdm2 and p53 protein expression in NSCLC patients [14].

It is currently clear that there are multiple forms of mdm2 proteins expressing different combinations of mdm2 epitopes, probably reflecting diversely spliced MDM2mRNAs [23]. Thus the monoclonal antibodies used in particular studies might fail to detect mdm2 protein in some cells. The second possible reason for the discrepancy between *MDM2* gene amplification and protein expression might be related to the methodology used and to the relatively small series of patients in particular studies.

In conclusion, presented results suggest that mdm2 protein accumulation in NSCLC cells does not necessarily result from *MDM2* gene amplification but might also be related to other mechanisms, such as increased transcription of MDM2mRNA or enhanced mdm2 protein translation. Future analyses are needed to clarify this issue.

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